JOURNAL OF CLINICAL MICROBIOLOGY, July 2005, p. 3463–3466 Vol. 43, No. 7
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.7.3463–3466.2005

Use of PCR Analyses To Define the Distribution of *Ralstonia* Species Recovered from Patients with Cystic Fibrosis

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Received 24 February 2005/Accepted 1 April 2005

Two new PCR assays (for *Ralstonia* species and *Ralstonia respiraculi*), together with previously published PCR assays, were used to assess *Ralstonia* isolates recovered from 111 cystic fibrosis patients. *Ralstonia mannitolilytica* accounted for 46% of isolates, while *R. respiraculi* and *Ralstonia pickettii* accounted for 19% and 18%, respectively. *Ralstonia basilensis* and *Ralstonia metallidurans*, species not previously recovered from human samples, were also identified.

Persons with cystic fibrosis (CF) are particularly susceptible to pulmonary infections with a wide range of bacteria (11). Typical pathogens include *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and members of the *Burkholderia cepacia* complex (11, 16). During the last decade, many other gram-negative, nonfermenting bacterial species, including *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *Pandoraea* sp., have also been recovered from CF patients' sputum (1, 2, 4, 9). While the frequency of infection with these species is relatively low and their clinical significance unclear, they present a significant challenge to diagnostic laboratories, as they are difficult to identify and often misidentified as *B. cepacia* complex.

Among the unusual organisms recovered from CF patients are several *Ralstonia* species. Currently, the genus *Ralstonia* consists of 14 validly described species. Among these, *Ralstonia pickettii*, *Ralstonia mannitolilytica*, *Ralstonia gilardii*, *Ralstonia paucula*, *Ralstonia taiwanensis*, *Ralstonia insidiosa*, and *Ralstonia respiraculi* have been recovered from various clinical sources, including respiratory secretions of CF patients. However, because of the rapidly changing taxonomy of this genus and the lack of rapid and reliable methods for species identification, the occurrence and clinical role of *Ralstonia* sp. have not been systematically investigated. We have recently described sensitive and specific PCR assays for the identification of several of these species, including *R. mannitolilytica*, *R. pickettii*, and *R. insidiosa* (3, 6). In the present study, we developed two additional PCR assays, one for the identification of all *Ralstonia* spp. (i.e., at the genus level) and another targeting *R. respiraculi*. We used these new assays, together with the previously published assays, to assess the distribution of *Ralstonia* species recovered from CF patients in the United States.

To design primers for the new PCR assays, we aligned relevant 16S rRNA gene sequences available in the GenBank database by using the MegAlign software package (DNAStar Inc., Madison, WI). These included 242 sequences from 14 validly described *Ralstonia* species, putative novel *Ralstonia* species, and several other phylogenetically related β-*Proteobacteria* and CF-relevant species. Primer pair RalGS-F and RalGS-R was designed to amplify a fragment of the 16S rRNA genes of all *Ralstonia* species, while primer pair Rres-F and Rres-R targeted species-specific signature sequences in variable regions V3 and V8a of the 16S rRNA gene of *R. respiraculi* (Table 1).

DNA for use in PCR assays was prepared from bacteria as described previously (15). DNA amplification was carried out in reaction mixtures containing 2.0 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 250 μM (each) deoxynucleoside triphosphate (Promega, Madison, WI), 0.4 μM of each primer, 1 U of *Taq* polymerase (Invitrogen, Carlsbad, California), 2 μl of DNA and adjusted to 25 μl by the addition of high-performance liquid chromatography-grade H_2O. For the *Ralstonia* genus-level PCR, amplification was carried out in a RapidCycler (Idaho Technologies Inc., Salt Lake City, Utah) thermocontroller. After denaturation at 95°C for 30 s, 30 amplification cycles were completed, each consisting of 20 s at 94°C, 20 s at 58°C, and 40 s at 72°C. A final extension of 1 min at 72°C was applied. For the *R. respiraculi*-specific PCR, amplification was carried out in a PTC-100 (MJ Research, Reno, NV) thermocycler. After an initial 2-min denaturation at 95°C, 26 amplification cycles were completed, each consisting of 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C, followed by an additional final extension of 5 min at 72°C. Negative-control PCRs with all reaction components except template DNA were included in every experiment. PCR assays employing each primer pair produced DNA products of the predicted sizes (Fig. 1).

In order to confirm PCR-based identification results, we performed comparative 16S rRNA gene sequence analysis. Nearly complete 16S rRNA genes (corresponding to positions 9 to 1500 in the *Escherichia coli* numbering system) were amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) with conserved primers UFPL and URPL as previously described (15). DNA sequencing was carried out with an Applied Biosystems ABI model 3700 sequencer and the protocols of the manufacturer (PE Applied Biosystems, Foster City, CA) by using the BigDye Terminator cycle sequencing ready reaction kit. Resultant sequences were visualized as chromatograms and manually edited using Chromas v.2.22 (Technelysium Pty. Ltd., Helensvale, Australia). Edited se-

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TABLE 1. 16S rRNA gene-based primer sets

Primer target	Annealing temp (°C)	Primer	Sequence (5′–3′)	Location[a]	Product size (bp)
All *Ralstonia* species	58	RalGS-F	CTGGGGTCGATGACGGTA	452–469	546
		RalGS-R	ATCTCTGCTTCGTTAGTGGC	979–998	
R. respiraculi	62	Rres-F	GTCCGGAAAGAAATGGCG	423–440	1,011
		Rres-R	TCCTTGCGGTTAGGCTACCC	1415–1434	

[a] Positions are relative to the 16S rRNA gene sequence of *Ralstonia solanacearum* (NC 003295).

quences were assembled using EditSeq (DNAStar Inc.) and identified by using BLASTN and comparison to sequences currently available in the NCBI database (www.ncbi.nlm.nih.gov/BLAST).

R. respiraculi strains were further assessed by whole-cell protein analysis. Strains were grown on tryptone soy agar for 48 h at 37°C, and preparation of whole-cell proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (18). Densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis using the Pearson product moment correlation coefficient were performed using the GelCompar 4.2 software package (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were identified by comparison to a database containing profiles of all *Burkholderia*, *Ralstonia*, *Alcaligenes*, *Achromobacter*, *Pandoraea*, and *Bordetella* species (2).

To assess the sensitivity and specificity of the new *Ralstonia* genus-specific PCR assay, we tested a set of 152 strains that included 92 strains representing 13 *Ralstonia* species and 60 strains representing 23 other CF-relevant species. More specifically, this set of 152 strains included 12 *Ralstonia* strains obtained from the BCCM/LMG bacteria collection (Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium): *Ralstonia eutropha* LMG 1199^T, *Ralstonia basilensis* LMG 18990^T, *Ralstonia solanacearum* LMG 2299^T, *R. gilardii* LMG 5886^T, *R. paucula* LMG 3245^T, *Ralstonia oxalatica* LMG 2235^T, *R. taiwanensis* LMG 19424^T, *R. taiwanensis* LMG 19464, *Ralstonia campinensis* LMG 19282^T, *R. campinensis* LMG 19285, *Ralstonia metallidurans* LMG 1195^T, and *R. metallidurans* LMG 18526. The remaining 80 *Ralstonia* strains were recovered from 80 CF patients and referred for analysis to the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan). These were identified as *Ralstonia* by either previously published PCR assays (3, 6) (33 *R. mannitolilytica* isolates, 17 *R. pickettii* isolates, and 2 *R. insidiosa* isolates) or 16S rRNA gene sequence analysis (13 *R. respiraculi* isolates, 8 *R. gilardii* isolates, 2 *R. metallidurans* isolate, 1 *R. taiwanensis* isolate, 1 *R. paucula* isolate, 1 *R. basilensis* isolate, and 2 strains classified as *Ralstonia*). The identification of the 13 *R. respiraculi* isolates in this set was further confirmed by whole-cell protein analysis (described above). Another 60 bacterial strains had been identified in previous studies (4, 5, 14, 15, 17, 20, 21) as belonging to 23 non-*Ralstonia* species. This group included 24 *B. cepacia* complex strains, 11 *Pandoraea* species strains, 7 *A. xylosoxidans* strains, 5 *Burkholderia gladioli* strains, 5 *P. aeruginosa* strains, 2 *S. maltophilia* strains, 2 *Herbaspirillum* species strains, and 1 strain each of *Bordetella bronchiseptica*, *H. influenzae*, *S. aureus*, and *Serratia marcescens*. Both the sensitivity and specificity of the novel *Ralstonia* genus-specific PCR assay were 100% (Table 2).

The sensitivity and specificity of the putative *R. respiraculi*-specific PCR were determined by testing 53 strains. This group included 13 strains identified as *R. respiraculi* by polyphasic testing that included 16S rRNA gene sequence analysis and whole-cell protein analysis. The 40 remaining strains represented 12 other *Ralstonia* species as well as *A. xylosoxidans* and all species within the *B. cepacia* complex and the genus *Pandoraea*. The sensitivity and specificity of this assay were 100% (Table 2).

To assess the distribution of *Ralstonia* species in persons with CF, isolates from an additional 31 *Ralstonia*-infected CF patients, which had been referred to the *Burkholderia cepacia* Research Laboratory and Repository during the course of the PCR validation studies, were combined with the 80 *Ralstonia* CF isolates described above to provide a total of 111 isolates from 111 CF patients. These patients received care in 56 CF care centers in 24 U.S. cities. All isolates had been identified as *Ralstonia* species by using previously published PCR assays

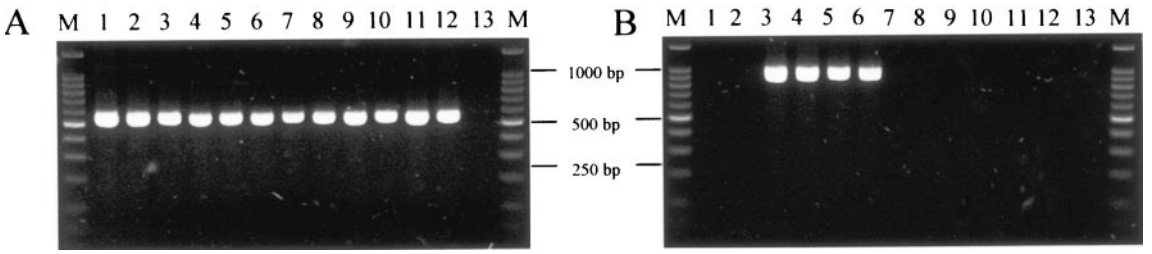

FIG. 1. PCR analysis of *Ralstonia respiraculi*, *Ralstonia* species, and CF-relevant bacteria. (A) PCR using *Ralstonia* genus-specific primers RalGS-F and RalGS-R. (B) PCR using *R. respiraculi*-specific primers Rres-F and Rres-R. Lanes M, reference markers; lanes 1, *R. mannitolilytica*; lanes 2, *R. pickettii*; lanes 3 to 6, *R. respiraculi*; lanes 7, *R. gilardii*; lanes 8, *R. insidiosa*; lanes 9, *R. paucula*; lanes 10, *R. metallidurans*; lanes 11, *R. taiwanenesis*; lanes 12, *R. eutropha*; and lanes 13, negative control (water).

TABLE 2. Sensitivities and specificities of PCR assays

PCR primer pair	Target	No. of PCR-positive strains/no. of tested strains			Sensitivity (%)	Specificity (%)
		Ralstonia spp.[a]	*R. respiraculi*[b]	Other[c]		
RalGS-F and RalGS-R	All *Ralstonia* species	79/79	13/13	0/60	100	100
Rres-F and Rres-R	*Ralstonia respiraculi*	0/24	13/13	0/16	100	100

[a] Includes *R. basilensis*, *R. campiensis*, *R. eutropha*, *R. gilardii*, *R. metallidurans*, *R. oxalatica*, *R. paucula*, *R. solanacearum*, and *R. taiwanensis* isolates from the BCCM/LMG culture collection and *R. gilardii*, *R. insidiosa*, *R. mannitolilytica*, *R. pickettii*, *R. respiraculi*, and an unidentified *Ralstonia* sp. recovered from CF sputum cultures (see the text).

[b] CF sputum isolates identified by polyphasic testing, including 16S rRNA gene sequence analysis.

[c] "Other" includes strains representing 23 non-*Ralstonia* species.

(specific for *R. pickettii*, *R. mannitolilytica*, or *R. insidiosa*), the novel *R. respiraculi* and *Ralstonia* genus-level PCR assays described above, or whole-cell protein and 16S rRNA gene sequence analysis (for species for which no PCR assay is available). The distribution of *Ralstonia* species among these patients is shown in Table 3. Two isolates were clearly identified as *Ralstonia* species based on whole-cell protein and 16S rRNA gene sequence analysis but could not be placed definitively into an existing species within this genus.

This distribution confirms the trend observed in a previous, smaller-scale study (6) in which *R. mannitolilytica* was found to be the predominant *Ralstonia* species recovered from respiratory secretions of CF patients, followed by *R. pickettii* and *R. respiraculi*. The present study also reiterates the fact that other *Ralstonia* species, including *R. gilardii*, *R. insidiosa*, and *R. paucula*, can be recovered from respiratory secretions of CF patients. Surprisingly, we also identified two *R. metallidurans* strains and a single *R. basilensis* strain. Both of these species are environmental organisms known for their metal resistance and ability to degrade a wide range of recalcitrant xenobiotics (12, 19). To our knowledge, neither species has been previously reported to be involved in human or veterinary infections. Whether these species were causing active infection in these patients or merely transiently colonizing their respiratory tracts remains unclear in the absence of clinical data. Nevertheless, the recovery of these species again highlights the facts that unusual microorganisms may be recovered from the respiratory secretions of CF patients (2) and that additional studies of the disease-causing potential of such species are needed.

Although considerable efforts have been made over the past several years to gain a better understanding of the biodiversity of *Ralstonia* species recovered from various ecological niches, including the respiratory tracts of CF patients (3, 7), strains that remain refractory to identification despite the use of state-of-the-art methods continue to be found. In the present study, we identified two strains that, based on comparative sequence analysis of the 16S rRNA gene sequence, clearly belong to the genus *Ralstonia*. Analysis of their protein profiles clearly suggested that they belong to two novel *Ralstonia* species. More in-depth polyphasic analyses will be required to determine the taxonomic status of these strains.

TABLE 3. Distribution of *Ralstonia* species recovered from 111 infected CF patients

Ralstonia species	No. of patients (%)
R. mannitolilytica	51 (46.0)
R. respiraculi	21 (18.9)
R. pickettii	20 (18.0)
R. gilardii	10 (9.0)
R. insidiosa	2 (1.8)
R. metallidurans	2 (1.8)
R. basilensis	1 (0.9)
R. paucula	1 (0.9)
R. taiwanensis	1 (0.9)
Indeterminate	2 (1.8)

Identification of *Ralstonia* species is difficult, and misidentification as *Pseudomonas fluorescens* or *B. cepacia* complex is frequent (8, 10, 13). Therefore, identification of *Ralstonia* species based on conventional methods should be confirmed with molecular (PCR-based) assays. We have previously described PCR assays for the identification of *R. mannitolilytica*, *R. pickettii*, and *R. insidiosa* (3, 6), and as is the case with the assays developed in the present study, these showed excellent sensitivity and specificity. Thus, reliable (i.e., sensitive and specific) PCR-based assays are now available for the genus-level identification of *Ralstonia* species as a whole, as well as for the species-level identification of *R. insidiosa* and the *Ralstonia* species most frequently recovered from patients with CF (i.e., *R. mannitolilytica*, *R. pickettii*, and *R. respiraculi*). Accurate identification of these species is a necessary prerequisite to further studies aimed at determining the clinical relevance of these species to CF.

Nucleotide sequence accession numbers. All 16S rRNA gene sequences generated in this study were deposited in GenBank under accession numbers AY860224 through AY860251.

This work was supported by a grant (to J.J.L.) from the Cystic Fibrosis Foundation. T.C. and P.V. are indebted to the Fund for Scientific Research—Flanders (Belgium) for a position as postdoctoral fellow and for research grants, respectively.

We acknowledge the generosity and cooperation of participating CF centers and microbiology laboratories for the submission of clinical isolates.

REFERENCES

1. **Burns, J. L., J. Emerson, J. R. Stapp, D. L. Yim, J. Krzewinski, L. Louden, B. W. Ramsey, and C. R. Clausen.** 1998. Microbiology of sputum from patients at cystic fibrosis centers in the United States. Clin. Infect. Dis. **27:**158–163.
2. **Coenye, T., J. Goris, T. Spilker, P. Vandamme, and J. J. LiPuma.** 2002. Characterization of unusual bacteria isolated from respiratory secretions of cystic fibrosis patients and description of *Inquilinus limosus* gen. nov., sp. nov. J. Clin. Microbiol. **40:**2062–2069.
3. **Coenye, T., J. Goris, P. De Vos, P. Vandamme, and J. J. LiPuma.** 2003. Classification of *Ralstonia pickettii*-like isolates from the environment and clinical samples as *Ralstonia insidiosa* sp. nov. Int. J. Syst. Evol. Microbiol. **53:**1075–1080.
4. **Coenye, T., L. Liu, P. Vandamme, and J. J. LiPuma.** 2001. Identification of *Pandoraea* species by 16S ribosomal DNA-based PCR assays. J. Clin. Microbiol. **39:**4452–4455.
5. **Coenye, T., E. Mahenthiralingam, D. Henry, J. J. LiPuma, S. Laevens, M. Gillis, D. P. Speert, and P. Vandamme.** 2001. *Burkholderia ambifaria* sp. nov.,

a novel member of the *Burkholderia cepacia* complex comprising biocontrol and cystic fibrosis-related isolates. Int. J. Syst. Evol. Microbiol. **51:**1481–1490.
6. **Coenye, T., P. Vandamme, and J. J. LiPuma.** 2002. Infection by *Ralstonia* species in cystic fibrosis patients: identification of *R. pickettii* and *R. mannitolilytica* by polymerase chain reaction. Emerg. Infect. Dis. **8:**692–696.
7. **Coenye, T., P. Vandamme, and J. J. LiPuma.** 2003. *Ralstonia respiraculi* sp. nov., isolated from the respiratory tract of cystic fibrosis patients. Int. J. Syst. Evol. Microbiol. **53:**1339–1342.
8. **De Baere, T., S. Steyaert, G. Wauters, P. Des Vos, J. Goris, T. Coenye, T. Suyama, G. Verschraegen, and M. Vaneechoutte.** 2001. Classification of *Ralstonia picketii* biovar 3/′thomasii' strains (Pickett 1994) and of new isolates related to nosocomial recurrent meningitis as *Ralstonia mannitolytica* sp. nov. Int. J. Syst. Evol. Microbiol. **51:**547–558.
9. **Denton, M., and K. G. Kerr.** 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clin. Microbiol. Rev. **11:**57–80.
10. **Gilligan, P.** 1995. *Pseudomonas* and *Burkholderia*, p. 509–532. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
11. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35–51.
12. **Goris, J., P. De Vos, T. Coenye, B. Hoste, D. Janssens, H. Brim, L. Diels, M. Mergeay, K. Kersters, and P. Vandamme.** 2001. Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov., and *Ralstonia basilensis* Steinle *et al.* 1998 emend. Int. J. Syst. Evol. Microbiol. **51:**1773–1782.
13. **Henry, D. A., E. Mahenthiralingam, P. Vandamme, T. Coenye, and D. P. Speert.** 2001. Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. J. Clin. Microbiol. **39:**1073–1078.
14. **LiPuma, J. J., B. J. Dulaney, J. D. McMenamin, P. W. Whitby, T. L. Stull, T. Coenye, and P. Vandamme.** 1999. Development of rRNA-based PCR assays for identification of *Burkholderia cepacia* complex isolates recovered from cystic fibrosis patients. J. Clin. Microbiol. **37:**3167–3170.
15. **Liu, L., T. Coenye, J. L. Burns, P. W. Whitby, T. L. Stull, and J. J. LiPuma.** 2002. Ribosomal DNA-directed PCR for identification of *Achromobacter* (*Alcaligenes*) *xylosoxidans* recovered from sputum samples from cystic fibrosis patients. J. Clin. Microbiol. **40:**1210–1213.
16. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194–222.
17. **Mahenthiralingam, E., J. Bischof, S. K. Byrne, C. Radomski, J. E. Davies, Y. Av-Gay, and P. Vandamme.** 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. J. Clin. Microbiol. **38:**3165–3173.
18. **Pot, B., P. Vandamme, and K. Kersters.** 1994. Analysis of electrophoretic whole-organism protein fingerprints, p. 493–521. *In* M. Goodfellow and A. G. J. O'Donnell (ed.), Chemical methods in prokaryotic systematics. Wiley & Sons Limited, Chichester, United Kingdom.
19. **Steinle, P., G. Stucki, R. Stettler, and K. W. Hanselmann.** 1998. Aerobic mineralization of 2,6-dichlorophenol by *Ralstonia* sp. strain RK1. Appl. Environ. Microbiol. **64:**2566–2571.
20. **Whitby, P. W., K. B. Carter, J. L. Burns, J. A. Royall, J. J. LiPuma, and T. L. Stull.** 2000. Identification and detection of *Stenotrophomonas maltophilia* by rRNA-directed PCR. J. Clin. Microbiol. **38:**4305–4309.
21. **Whitby, P. W., L. C. Pope, K. B. Carter, J. J. LiPuma, and T. L. Stull.** 2000. Species-specific PCR as a tool for the identification of *Burkholderia gladioli*. J. Clin. Microbiol. **38:**282–285.